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Enalapril treatment increases T cell number and promotes polarization towards M1-like macrophages locally in diabetic nephropathy

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ABSTRACT

Diabetic nephropathy (DN) is a serious complication of longstanding diabetes affecting up to 30% of all diabetes patients and is the main cause of end-stage kidney disease globally. Current standard treatment *e.g.* ACE-inhibitors like enalapril merely offers a delay in the progression leading to DN. Herein, we describe in two preclinical models evidence to local effects on the inflammatory signatures after intervention treatment with enalapril which provides enhanced understanding of the mechanism of ACE inhibitors.

Enalapril transiently reduced albuminuria in both the db/db and the STZ-induced DN models with established disease, without modulating the HbA1c%. Albuminuria was strongly associated with loss of leukocytes, particularly B cells, but also of sub-populations of macrophages and $CD4^+$ T cells. The remaining kidney macrophages were polarized into a M2-like sub-population with reduced surface expression of the M1-like macrophage marker CD11c and enhanced expression of galectin-3. Enalapril treatment counteracted the reduction of leukocytes in the diabetic kidney towards levels noted in the non-diabetic kidney. Particularly, a subset of macrophages was increased and a clear expansion of $CD4^+$ and $CD8^+$ T cells was observed. However, enalapril failed to modulate the B cell compartment. Interestingly, enalapril treatment resulted in a re-polarization of the macrophages towards a M1-like phenotype characterized by elevated levels of CD11c with moderate down-regulation of the M2 marker galectin-3.

The data demonstrate that ACE inhibition in pre-clinical models of DN shows a transient beneficial effect on albuminuria which is unexpectedly associated with restoration of T cells and M1-like macrophages in the kidney.

1. Introduction

Diabetic nephropathy (DN) is a serious complication of type 2 diabetes (T2D) with a prevalence of 30% of all diabetes patients [1]. DN is the main kidney disease leading to end stage renal disease (ESRD) and hence requirement for transplantations worldwide [1]. The disease is characterized by morphological and ultrastructural changes in the kidney including glomerular basement membrane thickening, mesangial expansion, nodular increase in mesangial matrix, glomerulosclerosis and finally interstitial fibrosis [2]. These modulations in the kidney tissue disrupt the filtration barrier, which lead to marked increase of albumin in the urine (albuminuria) and cause a decline in glomerular filtration rate (GFR) [2]. First line therapy in DN patients is angiotensin converting enzyme (ACE) inhibitors like enalapril [3]. These agents are thought to work mainly by lowering global and local blood pressure thereby reducing the pressure on the kidney. However, enalapril does have additional unclear kidney protective properties in addition to blood pressure modulation as other blood pressure reagents with higher efficacy fail to protect the kidney from development of albuminuria [4]. Angiotensin II is known to have pro-inflammatory properties and thus additional beneficial effect of enalapril might be due to a reduction of inflammation [5].

Emerging evidence suggests inflammation to play essential roles in the pathogenesis of T2D and diabetic complications including DN [6–13]. Infiltrating macrophages have been found in both diabetic kidneys from experimental animal models and human DN renal biopsies [12–14]. In human DN, macrophages and T cells accumulate in the glomeruli and interstitium, even in the early stages of the disease when the patient only shows minimal clinical symptoms [10,11]. These recruited inflammatory cells secrete pro-inflammatory cytokines in the renal environment triggering the release of a spectrum of chemokines along with the increased expression of adhesion molecules on resident renal cells [15]. This local inflammatory milieu further amplifies the migration of leukocytes initiating signaling cascades in the kidney tissue leading to detrimental effector functions considered to contribute to the clinical symptoms.

Macrophages are a heterogeneous population of cells. They express various levels of macrophage markers such as F4/80 and CD68, depending

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on local environmental factors or tissue of origin [16,17]. The local environment provides macrophages with various stimuli creating an array of tissue specific distinct cells. The macrophages activated by inflammatory stimuli such as IFN-γ, LPS, and TNF-α, polarize towards a classical M1-like phenotype, contributing with pro-inflammatory cytokines such as IL-12 and IFN-γ [18]. The alternatively activated M2-like phenotype, induced by Th2 cytokines such as IL-4 and IL-13 as well as galectin-3, contributes with IL-4, IL-10 and TGF-β production to tissue repair and remodeling in the resolution of an inflammatory response [19,20]. However, under certain conditions with unresolved chronic inflammation, an excessive activation of the M2 macrophages initiates pathological wound healing processes, resulting in irreversible fibrosis, tissue destruction and progressive chronic disease [21]. Recently, we described that in the db/db model of T2D, the local islet invading macrophages displays a M1-like phenotype in early disease, which was in the chronic stage of T2D re-polarized towards a M2-like phenotype also in the systemic compartment [22].

Macrophages account for the majority of leukocytes found in the kidney in DN and are believed to be central players in the DN pathogenesis [12,23]. Recently, several studies have investigated the state of activation and the phenotype of macrophages in the nephrotic kidney showing considerable phenotypic changes during disease progression. Macrophages infiltrating the kidney in early fibrotic kidney disease are predominantly of the M1 pro-inflammatory phenotype contributing to renal injury [24–26]. These macrophages subsequently undergo a local shift towards M2 macrophages which are the predominate phenotype during the recovery and repair phase and are suggested to be the main players responsible for subsequent development of fibrosis at the later stage of the disease [25]. Recently, a study by Fujiu et al. confirmed the previous findings by identifying two distinct macrophage subsets based on the expression of CD11b and F4/80 with different gene expression profiles and mutually exclusive functions [27]. The $CD11b^{+}F480^{low}$ subset accumulated at an early time point inducing apoptosis and tissue destruction whereas the $CD11b^{+}F480^{hi}$ macrophage subset increased at a later stage and was responsible for tissue remodeling and fibrosis [27].

Galectin-3 (the Mac-2 antigen), a β-galactoside-binding soluble lectin is highly expressed on activated macrophages and is well known for its immunoregulatory functions [28,29]. Galectin-3 positive macrophages are increased in the systemic splenic compartment in the db/db T2D model at the age when significantly enhanced albuminuria and mesangial cell expansion resembling DN are present [22,30]. Galectin-3 has been shown to participate in M2-polarization and to promote extensive fibrosis [31–33]. This is believed to be due to the ability of strengthen TGF-β signaling associated with tissue remodeling [34]. Elevated galectin-3 plasma levels have been shown in end stage renal disease [35].

Since growth factors belonging to the TGF-β superfamily play an essential role in renal fibrosis and macrophages are suggested to be among the main producers of TGF-β [21], we evaluated whether TGF-β production and galectin-3 expression in macrophage subsets are increased in the kidney of the db/db and STZ diabetic nephropathy mouse models. The STZ model shows significant β-cell deficiency, however it lacks the autoimmune component noted in human T1D nephropathy, while the db/db model lacking a functional leptin-receptor shows rapid weight gain, obesity and pronounced insulin resistance. Further, we addressed if a certain macrophage population was responsible for the galectin-3 expression. Finally, we evaluated the treatment effect of the first line therapy – the ACE inhibitor enalapril, which is administrated to diabetic nephropathy patients – on the inflammatory compartment in the diabetic kidney.

Taken together, a profile characterized by elevated expression of galectin-3 and TGF-β in concert with the reduced presence of M1-macrophages, B and T cells was demonstrated in the diabetic kidney when the albuminuria levels were significantly increased. Enalapril treatment promoted a longer lived M1-like macrophage phenotype and local increase in the number of $CD4^{+}$ T cells, partial down-regulation of galectin-3, but no modulation of TGF-β expression. These data suggest that the observed effect of ACE inhibition on albuminuria might be due to partial restoration of the M1-like phenotype of macrophages. Specific treatment regimens targeting the balance of M1–M2 macrophages might provide novel therapeutic openings in the treatment of diabetic nephropathy.

2. Materials and methods

2.1. Animals

Male db/db mice 6–8 weeks of age were purchased from Charles River, Belgium and fed Altromin 1324 food pellets ad libitum. From weeks 8–10 of age, one group of mice were given the enalapril treatment (5 mg/kg added to food pellets by Altromin) in the standard Altromin 1324 food pellets ad libitumuntil 20–22 weeks of age when the experiment was terminated. 129SV mice were purchased from Charles River, Germany at 8 weeks of age. At 9 weeks of age the mice were given streptozotocin (STZ) injections IP at a dose of 125 μg/kg on days 1 and 4. In two weeks post STZ treatment, blood glucose measurements were conducted and mice showing elevated blood glucose above 16 mM were included in the experiment. In six weeks post the initial STZ injection, the enalapril treatment (5 mg/kg added to food pellets by Altromin) was started which lasted until termination 12 weeks later. All *in vivo* experiments were performed at Phenos GmbH, Hannover, Germany under ethical approval from the national regulatory authorities that included treatment of diabetic animals with therapeutic agents like the ACE inhibitors.

2.2. Blood glucose measurement and HbA1c

Evaluation of blood glucose levels was performed once weekly using a Contour glucometer from Bayer, Leverkusen, Germany as indicated by the manufacturer. HbA1c% measurements were performed every four weeks by an Olympus AU400 using standard reagents.

2.3. Measurement of albuminuria

Albuminuria was determined at the start of experiment (db/db mice at 10 weeks of age and STZ mice 6-weeks post STZ treatment) as baseline value, mid-point 6-weeks into the experiment and terminal at 12-weeks post start of experiment.

For urine collection, the mice were placed in metabolic cages for 15–16 h overnight. During the stay in the metabolic cages the animals had free access to food and water, but did not have bedding or enrichment. Albumin levels were measured in the urine samples using the Mouse Albumin ELISA Kit (Bethyl Lab, Hamburg, Germany) according to the manufacturer's instructions, and converted to 24 h urinary albumin excretion by dividing the exact number of hours in the metabolic cages and multiplying by 24.

2.4. Preparation of single cell suspension from mouse kidney

Kidneys were cut into small pieces and digested in media (RPMI with 5% serum) supplemented with 2 mg/ml collagenase II (Gibco) and 50 U/ml DNase (Sigma, St. Louis, MO, USA) by incubating for 40 min at 37 °C on a roller mixer. To release glomeruli from interstitium the samples were pipetted up and down before passing through a 100 μm cell strainer (BD Biosciences). The cell suspension was washed and erythrocytes were lysed with cell lysis buffer. To digest glomeruli, the preparation was further incubated in media supplemented with 0.5 mg/ml collagenase type II, 50 mg/ml dispase II (Sigma), 50 U/ml DNase and 0.075% trypsin (Gibco). The cells obtained were washed, resuspended in 1× HBSS without salts with 2 mM EDTA and incubated for 10 min on ice. In order to disrupt tubular structures the suspension

was passed 3 times through a needle (23 G). The cells were finally passed through a 40 μm cell strainer (BD).

2.5. Flow cytometry analysis

Flow cytometric analysis was performed according to standard procedures. Briefly, cells were first blocked for unspecific binding with anti-CD16/CD32 (BD Biosciences), followed by surface staining of CD11c (BD Biosciences), F4/80 (BioLegend), CD45 (eBioscience), B220 (BD Horizon), CD4 (BioLegend), CD8 (BD Biosciences). Cells were then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's description and then intracellularly stained for CD68, CD206 (both AbD Serotec), galectin-3 (BioLegend) and TGF-β (BioLegend). Samples were acquired on a FACS LSRFortessa, equipped with blue, red and violet lasers, followed by data analysis using FACSDiva software (BD Biosciences).

2.6. Determination of local kidney cytokine production

Protein homogenate was generated from frozen kidney tissue. Briefly, kidney was placed in a tissue lyser with lysis buffer [aprotinin (Sigma), leupeptin (Tocris) and pepstatin (Tocris)] followed by addition of Triton-X100 (Sigma) and finally centrifuged. Protein concentration was determined and equal amounts of protein were added to Proteome Profiler Mouse Angiogenesis Array Kit (R&D System). Relative expression of cytokines within the kidney tissue was expressed as signal detected over background signal for each protein analyzed.

2.7. Data analysis

Data were analyzed by 1-way ANOVA and Dunnett's post hoc test with the diabetic vehicle treated group as control. The level of significance was set at $p < 0.05$. All analyses were done using GraphPad Prism version 6.02.

3. Results

3.1. Development of diabetic nephropathy

To generate translational knowledge, the effect of enalapril was determined in an advanced STZ mouse model (mimicking T1D DN; hyperglycemia and with diminished β-cell mass) and the db/db mouse model (mimicking T2D DN; hyperphagic, obese, hyperglycemic and dyslipidemia) at a stage that resembles the human situation when enalapril treatment is initiated (Fig. 1A, B). Both models had been hyperglycemic for 4–5 weeks at the initiation of the enalapril treatment as indicated by the figure (Fig. 1A, B).

Both the db/db and the STZ-treated mice showed pronounced diabetes at the start of the experiment measured as elevated HbA1c% (Fig. 1C, E). The development of diabetes showed a progression during the experiment reaching 2.5 to 1.3-fold enhanced blood glucose levels at termination respectively (Fig. 1C, E). Treatment with enalapril had no effect on diabetes measured as HbA1c compared to untreated diabetic animals (Fig. 1C, E). The db/db mice had significantly enhanced albuminuria already at the start of the experiment with levels 5-fold higher than age-matched healthy db/+ mice (Fig. 1D). Interestingly, the db/db mice showed a 1.5-fold increase of albuminuria after 6 weeks, which then was reduced to levels below the starting value at termination of the experiment (Fig. 1D). Treatment with enalapril significantly reduced the albuminuria after 6-weeks of treatment (Fig. 1D). At termination of the experiment, the albuminuria levels in the enalapril treated group were at the same level as after 6-weeks treatment. However, as the untreated vehicle group showed a spontaneous reduction of albuminuria levels, there was no significant treatment effect of enalapril (Fig. 1D). At start of the STZ model experiment (6-weeks post STZ treatment), the albuminuria levels were the same in the STZ group as in the non-STZ mice (Fig. 1F). Similarly to the db/db mice, the STZ mice showed a transient increase of albuminuria with significant 2.5-fold increased levels at 6-weeks, which then declined to 1.5-fold the starting values at termination of the experiment (Fig. 1F). Treatment with enalapril significantly reduced the increase of albuminuria after 6 weeks of treatment (Fig. 1F). However, due to the spontaneous reduction of albuminuria at termination of the experiment, no significant effect of albuminuria was noted with enalapril after 12 weeks of treatment. Also in this model, the albuminuria level in the enalapril treated group was stable from 6–12 weeks of treatment.

These data show that in both T1D and T2D models of DN, a transient increase of albuminuria was noted with peak level of albuminuria at 16-weeks of age in db/db mice and 21-weeks of age in STZ treated animals. Enalapril treatment in mice experiencing hyperglycemia for a similar period showed no effect on development of diabetes, but significantly delayed the progression of albuminuria in both models.

3.2. Enalapril treatment restores leukocyte numbers in the diabetic kidney

With the presence of DN established in both the T1D and T2D DN models, the effect of enalapril on the local inflammatory milieu was determined at termination of the experiment measured as the total number of leukocytes in the db/db and STZ kidney compared to their healthy control group.

The previously described immune-compromised db/db mice showed a significant reduction in the total number of $CD45^+$ leukocytes compared to healthy db/+ littermates (Fig. 2A). Treatment with enalapril significantly increased the number of leukocytes present in the kidney to levels detected in the healthy db/+ littermates (Fig. 2A). Although reduced, there was no statistically significant reduction of leukocytes in the STZ treated mice (Fig. 2B). However, as noted in the db/db mice, enalapril treatment resulted in a significant increase in the number of leukocytes at termination of the experiment (Fig. 2B). Of interest, the total number of leukocytes regardless diabetes or diabetic nephropathy was 5-fold lower in the db/+ and db/db compared to the 129SV and STZ 129SV mice (Fig. 2A, B).

Taken together, in both T1D and T2D advanced pre-clinical DN a reduction of leukocytes was observed compared to healthy control mice. Enalapril treatment prevented the loss of leukocytes in both models and with STZ treatment the levels were even higher than in non-diabetic control mice.

3.3. Enalapril treatment results in increase of kidney $CD68^+F/80^+$ macrophages in db/db mice

Studies have shown a correlation between increased number of kidney macrophages and the severity of DN in patients [35–38]. Further, macrophages have been shown to be the first immune cells to enter the diabetic kidney [21,39,40]. Given the observed reduction of leukocytes in the advanced diabetic kidney, particularly in the db/db mice and the increase in numbers after enalapril treatment, the number of macrophages was determined (Fig. 3A–C).

In the db/db T2D model, the total numbers of both $CD68^+F4/80^+$ and $CD68^+F4/80^-$ macrophages were significantly reduced compared to healthy littermates (Fig. 3D, E). Enalapril treatment counteracted the loss of $CD68^+F4/80^+$ macrophages, while it had no effect on the $CD68^+F4/80^-$ macrophages (Fig. 3D, E). Interestingly, the number of macrophages in STZ treated mice was not significantly modulated compared to healthy control mice. Although a small trend to increase in both macrophage subsets was noted after enalapril treatment, no significant changes were demonstrated in the STZ mice after treatment (Fig. 3F, G).

Taken together, the results showed that enalapril treatment counteracted the loss of $CD68^+F4/80^+$ macrophages, but not of

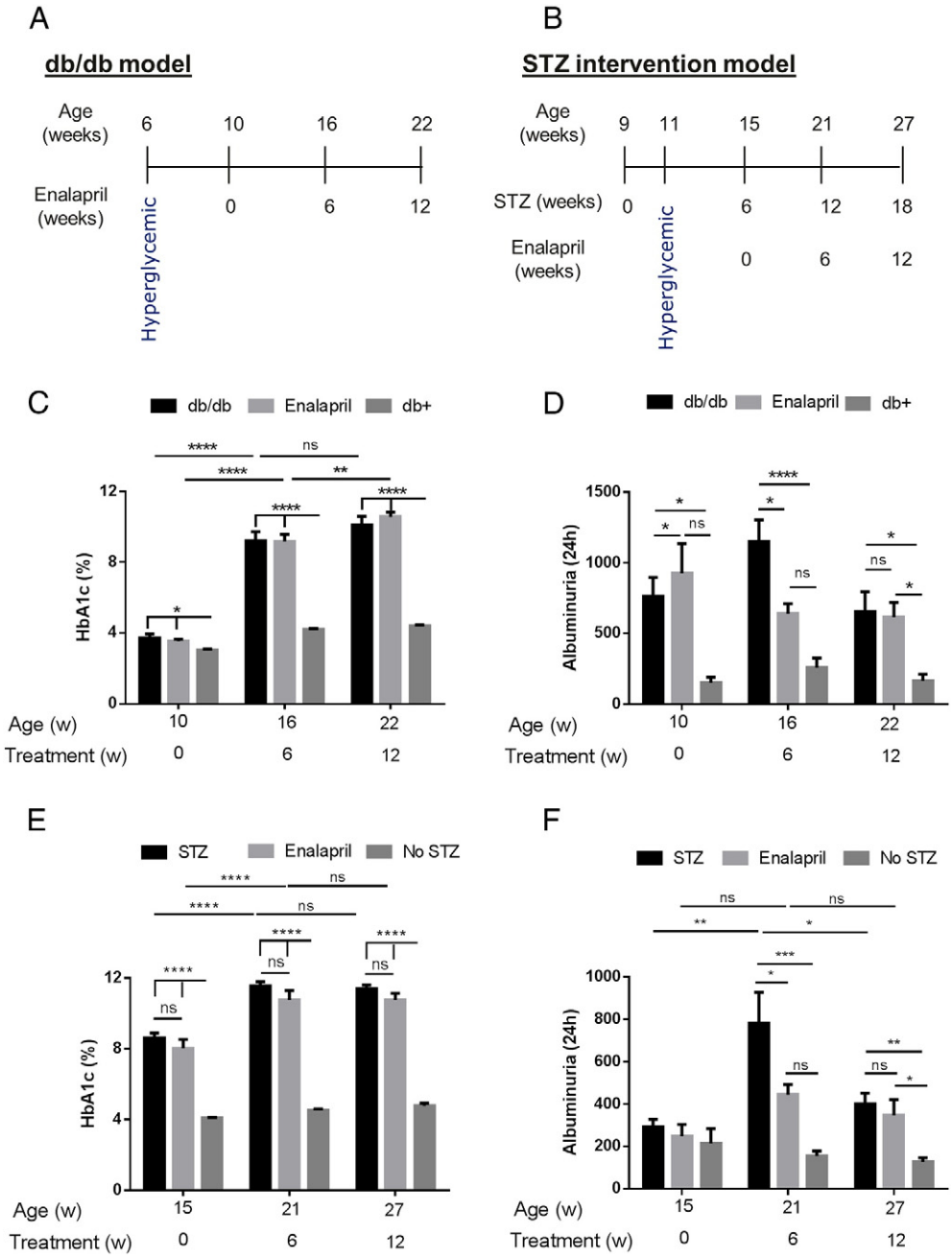

Fig. 1. Layout of the diabetic nephropathy models used in the study (A, B). HbA1c (C, E) and albuminuria (D, F) in db/db (C, E) and STZ (D, F) mice before and after 6 or 12 weeks of treatment with ACE inhibitor and their respective controls. ****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05.

$CD68^+F4/80^-$ macrophages in the T2D DN db/db model, whereas no statistical effect was observed on macrophage numbers in the T1D STZ model.

3.4. Enalapril promotes polarization of the macrophages towards a M1-like phenotype

To further evaluate if enalapril modulates not only the number of macrophages, but also the functional stage of the kidney derived macrophages, expression levels of markers associated with M1 and M2 polarization were determined (Fig. 4).

Kidney-derived macrophages from advanced DN in the db/db mice showed a significant reduction of the M1-associated CD11c marker both in the $CD68^+F4/80^+$ and $CD68^+F4/80^-$ subsets compared to healthy db/+ littermates (Fig. 5A, B). Treatment with enalapril significantly increased the expression of CD11c on both subsets, but particularly on $CD68^+F4/80^-$ macrophages in the db/db mice compared to untreated mice (Fig. 5A, B). In the STZ mice, a highly significant reduction of CD11c was noted on the $CD68^+F4/80^+$ macrophages, while the $CD68^+F4/80^-$ macrophages showed only a partial non-significant reduction (Fig. 5C, D). In contrast to the db/db model, no restoration of CD11c expression level was noted on $CD68^+F4/80^+$ macrophages in the STZ mice after enalapril treatment (Fig. 5C). However, the expression of CD11c on $CD68^+F4/80^-$ cells trended to be enhanced after enalapril treatment in the STZ mice compared to untreated STZ mice (Fig. 5D). A moderate reduction of the M2-associated marker CD206 was demonstrated in the $CD68^+F4/80^+$ macrophages in the db/db mice, whereas no modulation was noted in the $CD68^+F4/80^-$ macrophages (Fig. 5E, F). Enalapril treatment did not modulate the expression of CD206 in the db/db mice (Fig. 5E, F). A non-significant pattern suggesting an increase of CD206 expression on $CD68^+F4/80^+$ macrophages was noted in the STZ mice, while no modulation was noted in the $CD68^+F4/80^-$

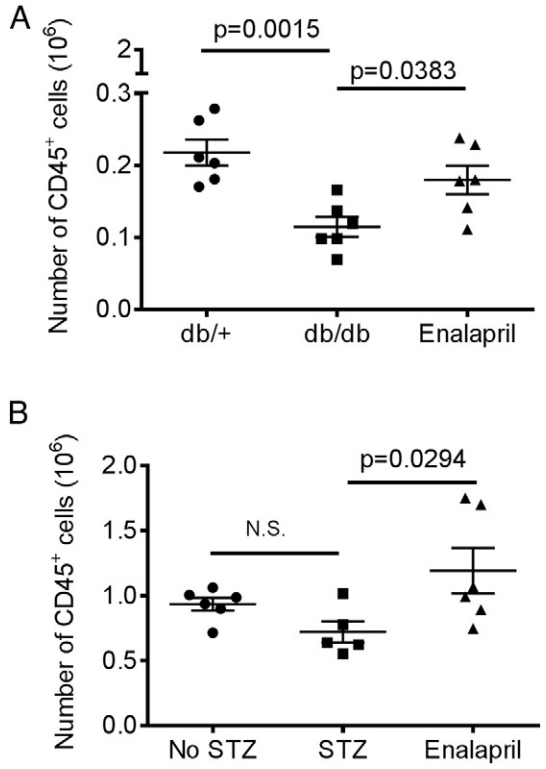

Fig. 2. Total number of $CD45^+$ cells in (A) db/db and (B) STZ kidneys with and without treatment with ACE inhibitor and their respective controls (mean ± SEM; n = 6).

macrophage subset (Fig. 5G, H). Enalapril treatment showed a non-significant trend to further increased CD206 expression compared to untreated STZ mice in the $CD68^+F4/80^+$ macrophages, resulting in a highly significantly enhanced expression compared to healthy control mice (Fig. 5G).

Taken together, these data revealed that the advanced diabetic nephropathy in pre-clinical mouse models is associated with a highly significant reduction of the classical M1-like macrophage phenotype while the M2 marker CD206 is only moderately modulated locally in the kidney. Interestingly, enalapril treatment promotes a polarization of the kidney macrophages towards an M1-like phenotype in a subset of macrophages in both db/db and STZ mice. Thus, ACE inhibition appears to influence the shift in M1/M2 polarization in macrophages overall favoring the classically activated M1-like pro-inflammatory phenotype of macrophages (CD11c), which is reduced with disease progression in mouse diabetic nephropathy while no significant modulation of the alternatively activated CD206 macrophages was noted.

3.5. Enalapril treatment partly counteracts polarization of macrophages into a galectin-3^+ phenotype

Galectin-3 is expressed by a subset of macrophages which has been shown to be a key driver of fibrosis in several chronic inflammatory disorders often associated with M2-like polarization [32,33,36]. As enalapril treatment in late pre-clinical diabetic nephropathy influenced the polarization of macrophages into a M1-like phenotype, the galectin-3 expression level on the kidney macrophages was determined as a measurement of a fibrotic macrophage signature (Fig. 4).

No modulation of galectin-3 expression was noted in $CD68^+F4/80^+$ macrophages in either the T2D db/db or in the T2D STZ model (Fig. 6A, C). In sharp contrast, a 2-fold increased galectin-3 expression level was noted in both the db/db and in the STZ model on $CD68^+F4/80^-$ macrophages (Fig. 6B, D). Treatment with enalapril in the db/db model did show a significant 50% reduction of the expression compared to the untreated db/db mice (Fig. 6B). A similar, but less pronounced non-significant 40% reduction of galectin-3 expression was noted on the $CD68^+F4/80^-$ macrophages in STZ-treated mice after enalapril administration (Fig. 6D). Of note, the galectin-3 expression in the db/db mice was significantly higher compared to the STZ mice (Fig. 6A–D).

These data show that enalapril partly counteracts the elevation of galectin-3 expression in the kidney derived $CD68^+F4/80^-$ macrophages in both models suggesting that ACE inhibition might provide beneficial effect on the fibrotic signature in M2-like macrophages.

3.6. Enalapril failed to modulate the expression of TGF-β in diabetic kidney macrophages

The pro-fibrotic property of galectin-3 is partly due to its ability to strengthen TGF-β signaling by stabilizing the TGF-β receptor and hence to promote myofibroblast differentiation [36]. As TGF-β has been shown to drive fibrosis in diabetic nephropathy and expression of galectin-3 was modulated in the model, the intrinsic TGF-β production in kidney macrophages was determined after enalapril treatment (Fig. 4).

Interestingly, neither the $CD68^+/F4/80^+$ nor the $CD68^+F4/80^-$ macrophages showed elevated levels of TGF-βI in the db/db mice (Fig. 6E, F). In contrast, the STZ treated $CD68^+F4/80^+$ macrophages showed a highly significant upregulation of TGF-β, while the $CD68^+F4/80^-$ only showed a trend towards upregulation (Fig. 6G, H). Enalapril treatment in both the db/db and the STZ model had no modulatory effect on the expression of TGF-β in the macrophages (Fig. 6E–H).

Taken together, the data demonstrate that intrinsic TGF-β expression in db/db kidney macrophages is not elevated compared to healthy mice, while STZ kidney derived macrophages showed a significantly higher expression level. Enalapril treatment is ineffective in modulating the intrinsic capacity to express TGF-β in the diabetic kidney macrophages.

3.7. Significant lymphocyte defects in the diabetic kidney are partly counteracted by enalapril treatment

A growing understanding of lymphocytes in diabetic nephropathy has been generated the last few years [37,38]. To address whether enalapril treatment affected the kidney associated trafficking of lymphocytes, B cell, $CD4^+$ and $CD8^+$ T cell numbers were determined.

In the db/db model, the overall number of lymphocytes was reduced. Particularly, the number of B cells in the diabetic db/db kidney was highly significantly reduced compared to healthy db/+ mice (Fig. 7A). Moreover, as in peripheral tissue *e.g.* spleen, the db/db mice showed a significant reduction of $CD4^+$ T cells in the diabetic kidney, while a non-significant reduction of $CD8^+$ T cells was noted (Fig. 7C, E). Similarly, a general reduction of lymphocytes was noted in the advanced STZ diabetic kidney. Although not significant, a 50% in reduction of absolute count of B cells was noted in the STZ diabetic animals compared to healthy non-STZ mice, while no modulation of T cell absolute numbers was noted (Fig. 7B, D, F). Treatment with enalapril had no effect on the B cell absolute count in neither of the models (Fig. 9A, B). In sharp contrast, the T cells were significantly affected by enalapril treatment. In db/db mice normalization to the levels recorded in healthy non-diabetic db/+ mice was noted (Fig. 7C, E). In the STZ mice, both the $CD4^+$ and the $CD8^+$ T cells showed pronounced expansion to levels clearly above the levels noted in healthy littermates (Fig. 7D, F).

These data show that the diabetic kidney is markedly lymphopenic compared to the healthy kidney. The reduction of B cells cannot be counteracted by ACE inhibition. In contrast, the T cell compartment and particularly the $CD4^+$ T cells responded to enalapril treatment by an increased accumulation in the kidney.

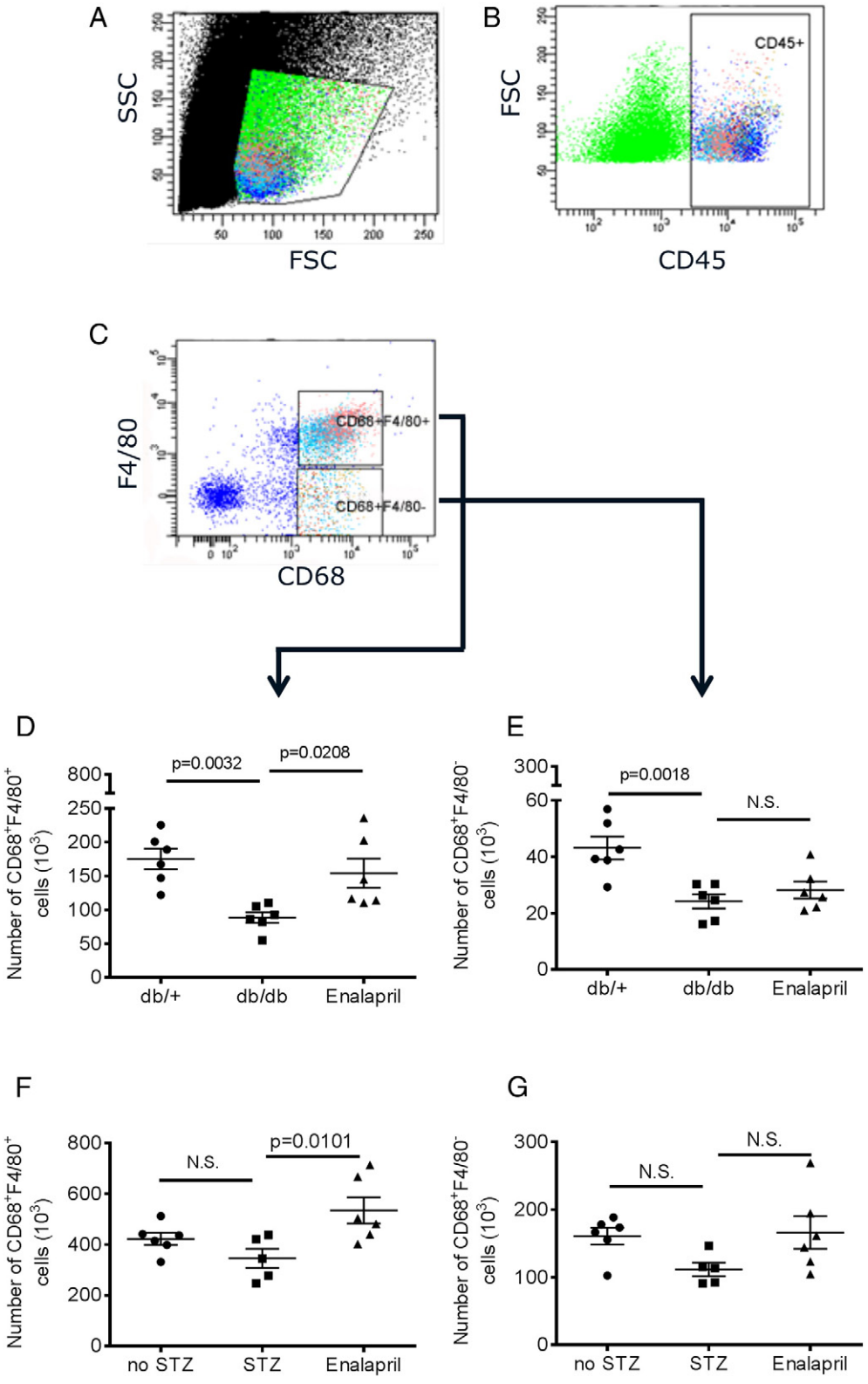

Fig. 3. Gating strategy to identify subsets of macrophages in the kidney (A–C). Total number of $CD68^{+}F4/80^{+}$ *vs.* $CD68^{+}F4/80^{-}$ macrophages in (D, E) db/db and (F, G) STZ kidneys with and without treatment with ACE inhibitor and their respective controls (mean ± SEM; n = 6).

3.8. The diabetic kidney expresses a modulated chemokine environment which is unaffected by enalapril

Local production of chemokines, cytokines and growth factors are known to modulate the presence of leukocytes in inflamed tissues [18, 39]. To address if enalapril treatment modulated the chemokine signature in the diabetic kidney, proteins in whole kidney homogenates were analyzed.

Several cytokine and chemokines were modulated in the diabetic db/db and STZ kidneys. However, only CXCL16 was significantly

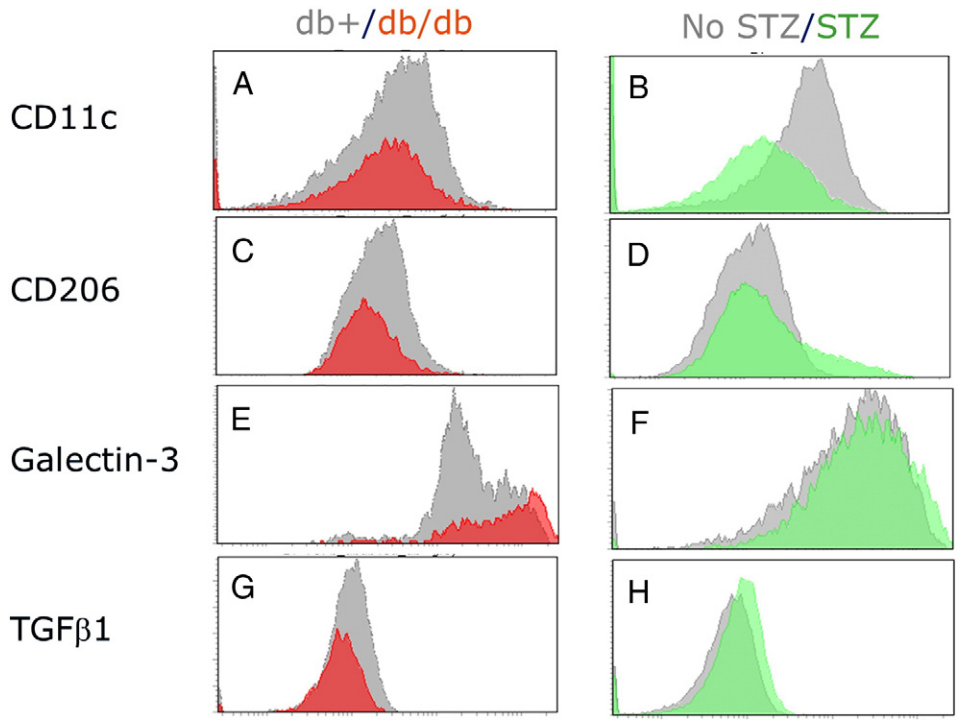

Fig. 4. Representative mean fluorescence intensity (MFI) histograms for $CD68^{+}F4/80^{+}$ CD11c (A, B), CD206 (C, D), TGFβ1 (G, H) and $CD68^{+}F4/80^{-}$ Galectin-3 (E, F) comparing healthy db^{+} versus diabetic db/db kidneys (A, C, E, G) and healthy non-STZ and diabetic STZ-induced kidneys (B, D, F, H).

upregulated in both the db/db and the STZ diabetic kidneys compared to non-diabetic tissue (Fig. 8A, B). Notably, the levels of KC and fractalkine were both markedly higher in the db/db model than in the STZ model (Fig. 8C, D). In the db/db kidney, a non-significant reduction of KC was noted in the diabetic kidney compared to the healthy tissue (Fig. 8C). Of importance, treatment with enalapril significantly reduced the diabetes enhanced CXCL16 levels in the STZ model, but failed to do so in the db/db model (Fig. 8A, B). In contrast, enalapril treatment in the STZ model significantly enhanced the levels of both KC and fractalkine, while no treatment effect was noted in the db/db model (Fig. 8C–F).

Taken together, the data shows that the expression of cytokines and chemokines is modulated in diabetic nephropathy. Moreover, enalapril modulates the expression pattern but only in the STZ model.

4. Discussion

Prolonged exposure to elevated glucose concentrations or frequent rapid fluctuations causes significant stress to the kidney [40]. Over time this leads to structural changes in the kidney that are manifested in human subjects as loss of podocytes, mesangial cell expansion, glomerulosclerosis and interstitial fibrosis [40]. With the change in life style during the last decades, the number of diabetes patients has increased significantly and with that the number of patients suffering from the diabetic complication diabetic nephropathy [1]. Although several lines of therapies exist including the ACE inhibitors, the efficacy of them is only partial and may delay, but never revert the decline in kidney function [3]. This leads to an increased number of patients waiting for kidney transplantation associated with huge cost burdens for the society.

Several animal models of DN have together provided valuable information regarding pathophysiology, progression, implicated genes and proposed new therapeutic strategies [13]. The common for all pre-clinical animal models is that they fairly well mimic the early changes in the human disease, but fail to develop the later stages [13]. Despite this limitation, they have been instrumental for the emerging concept that DN indeed contains local modulation of the inflammatory signature [11,14,41,42]. In our models, we prolonged the standard time for diabetic nephropathy mouse models with an additional 4–7 weeks to further allow tissue modulations to take place. In these prolonged models, we unexpectedly noted a significant reduction of total leukocytes in the db/db model and a trend to reduction in the STZ model. This reduction in leukocytes was mainly due to a loss of macrophages and $CD4^{+}$ T cells in the db/db mice, B cells in both the db/db and the STZ mice and $CD8^{+}$ T cells in the STZ mice. Although surprising, the reduction of macrophages observed in the diabetic kidney is consistent with previous studies showing that the differentiation/polarization state of the macrophages was changed rather than the number of cells in the diabetic kidney [24]. These observations are in line with our previous observation that the initiation of diabetes is strongly associated with a M1-like phenotype which gradually transforms into a M2-like phenotype in late stage diabetes in the systemic compartment [22]. Polarization and differentiation of macrophages are difficult to address since macrophages rapidly undergo re-polarization depending on local signals [18]. Several markers have been used to identify M1-like and M2-like macrophages but only rarely can a clear M1 or M2 macrophage be described *in vivo*. It is however well accepted, and was recently described in a pre-clinical diabetic model, that among other markers, CD11c is upregulated on M1-like macrophages, whereas CD206 (the mannose receptor) is expressed on M2-like macrophages [19,20,22]. Interestingly, the macrophages present in the advanced db/db and STZ kidneys had highly significantly reduced expression of the M1 marker CD11c. Macrophages expressing CD11c have been shown to have a classical pro-inflammatory cytokine signature associated with elevated production capacity of IFN-γ, TNF-α and IL-1β [18,20,43]. Moreover, these cells are considered highly professional APC and are associated with elevated levels of MHC-II and several co-stimulatory molecules needed to promote strong lymphocyte responses [43]. We recently described that the systemic expression of these pro-inflammatory cytokines in the plasma is similar in healthy control animals and in late stage disease in the db/db model, whereas in the initiation phase the levels are increased [22]. Herein, we extend this observation, by describing that the local kidney levels of these cytokines

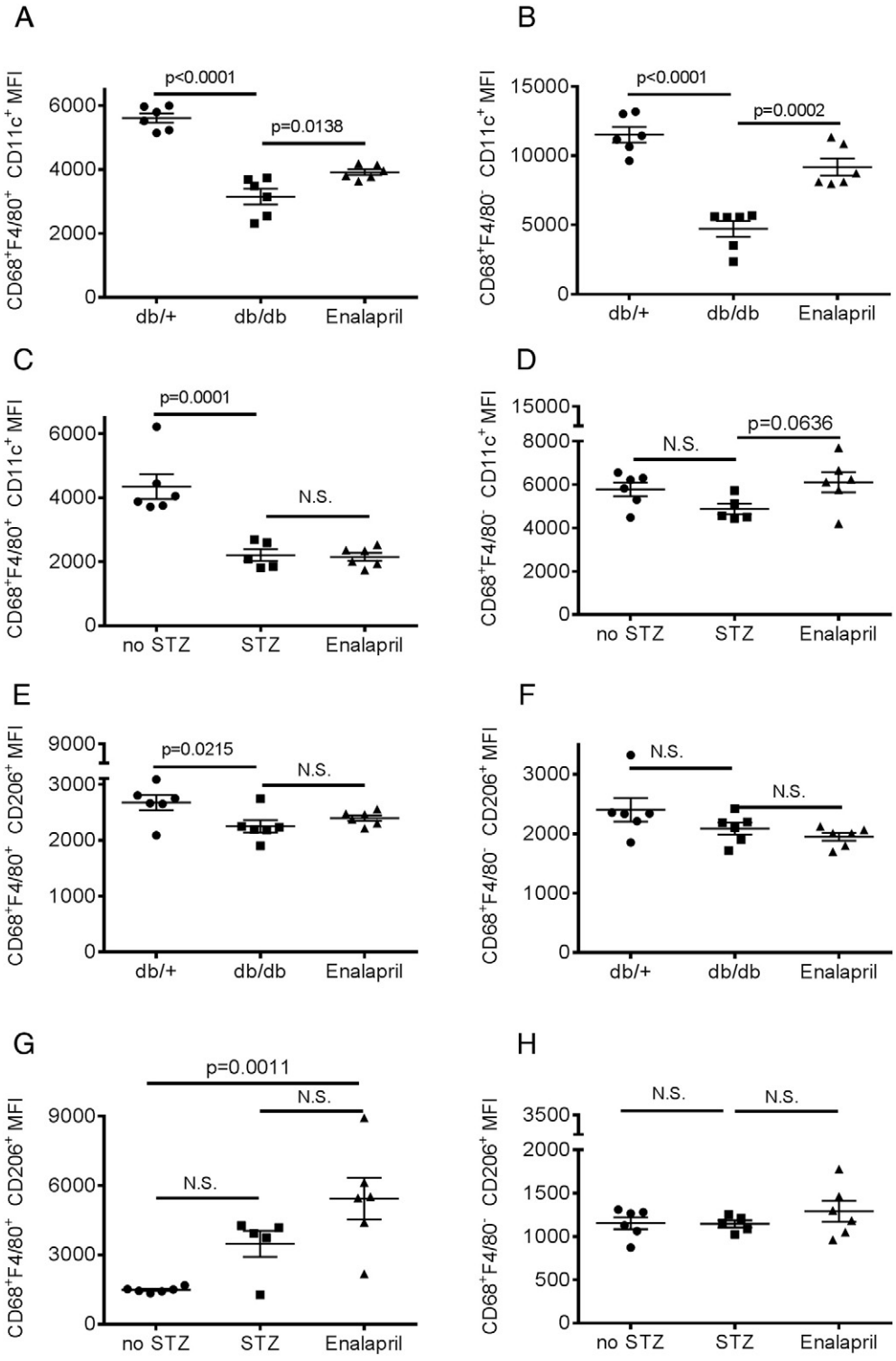

Fig. 5. Mean fluorescence intensity (MFI) showing CD11c (A–D) and CD206 (E–H) expression levels on $CD68^+F4/80^+$ *vs.* $CD68^+F4/80^-$ macrophages in db/db (A, B, E, F) and STZ (C, D, G, H) kidneys with and without treatment with ACE inhibitor and their respective controls (mean ± SEM; n = 6).

are low. This suggests that the prolonged exposure to glucose modulates the macrophages into a state with reduced capacity to produce pro-inflammatory cytokines associated with M1-like phenotype. Interestingly, in the db/db model, the kidney derived macrophages also showed the reduced expression of the M2-associated marker CD206, while a trend towards elevated levels were noted in the STZ macrophages. Li et al. recently showed that polarization towards alternatively activated M2-like phenotype in a high-fat diet (HFD) induced model was dependent on iNKT cellular activity [44]. In absence of these cells in the $Cd1^{-/-}$ mouse, HFD failed to induce these M2-like cells resulting in severe hyperglycemia and insulin tolerance [44]. db/db mice are known to be highly insulin resistant and display accelerated hyperglycemia [45]. In addition, these mice show a defect development of iNKT cells associated with significantly reduced numbers in peripheral tissues including the adipose tissue [46]. In contrast, STZ treated mice showed normal development of iNKT, but the cells do show reduced

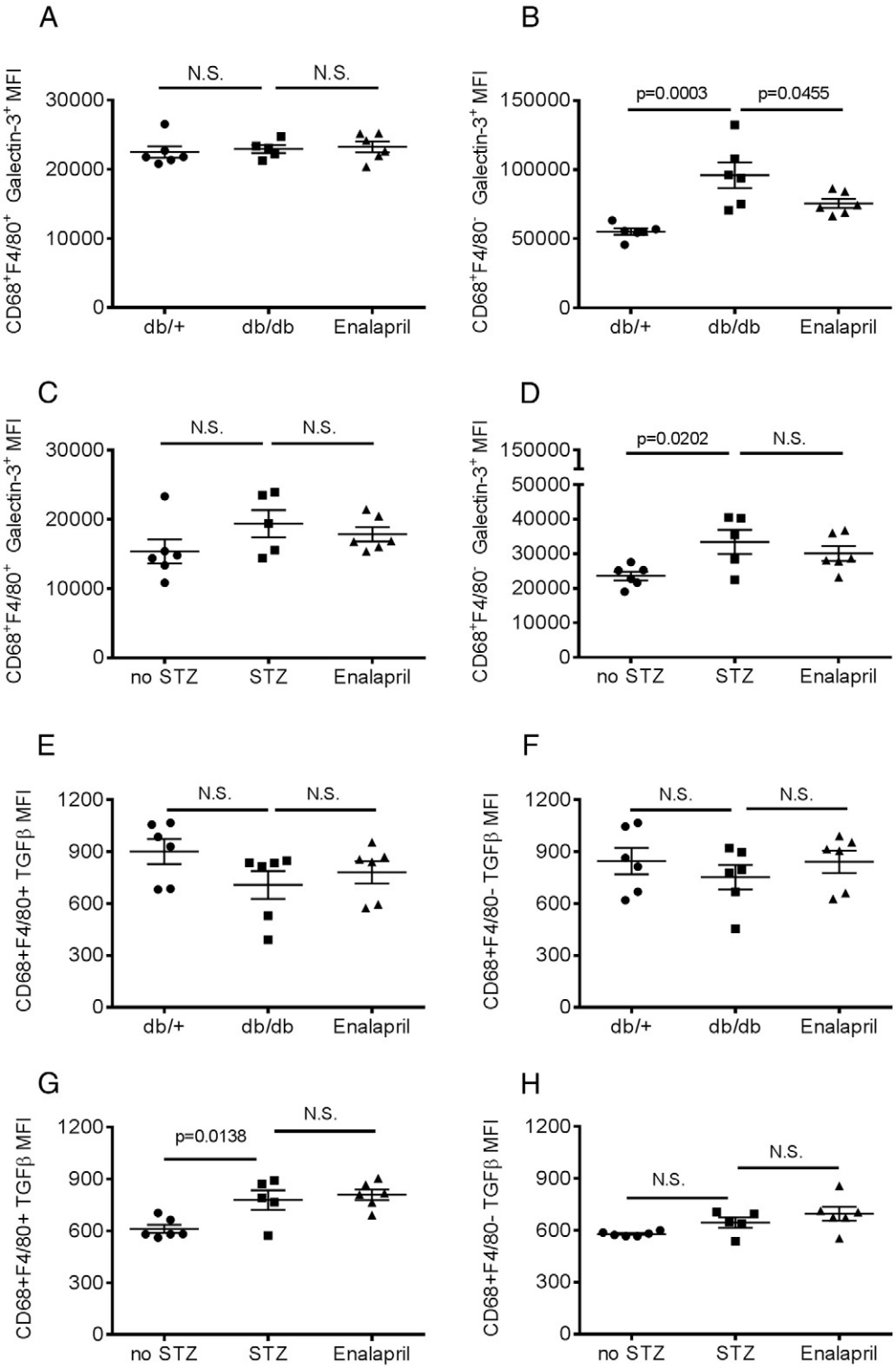

Fig. 6. Mean fluorescence intensity (MFI) showing galectin-3 (A–D) and TGFβ (E–H) expression levels on $CD68^+F4/80^+$ *vs.* $CD68^+F4/80^-$ macrophages in db/db (A, B, E, F) and STZ (C, D, G, H) kidneys with and without treatment with ACE inhibitor and their respective controls (mean ± SEM; n = 6).

capacity to produce Th2 cytokines like IL-4, while maintaining production of Th1 cytokines like IFNγ [47]. Hence the reduction of macrophages in the db/db mice and consequent partial loss of M2-like phenotype in the db/db mice could be dependent on the selective loss of iNKT cells in that strain, while the partial induction and long lived macrophage population in the STZ mice showing a mixed M1/M2 polarization could relate to the altered function of the cross-talk with the iNKT.

Late stage DN in humans is associated with excessive remodeling and fibrotic events [2]. M2 macrophages are well described to promote remodeling activity and fibrosis in other conditions and *in vitro* [18,20]. Our data with a relative shift from a M1-like towards M2-like macrophages suggests that the local leukocytes in the diabetic kidney resemble the phenotype known to initiate fibrotic events. This was further strengthened by the increased expression of galectin-3 on macrophages in both models. Galectin-3, a β-galactoside-binding

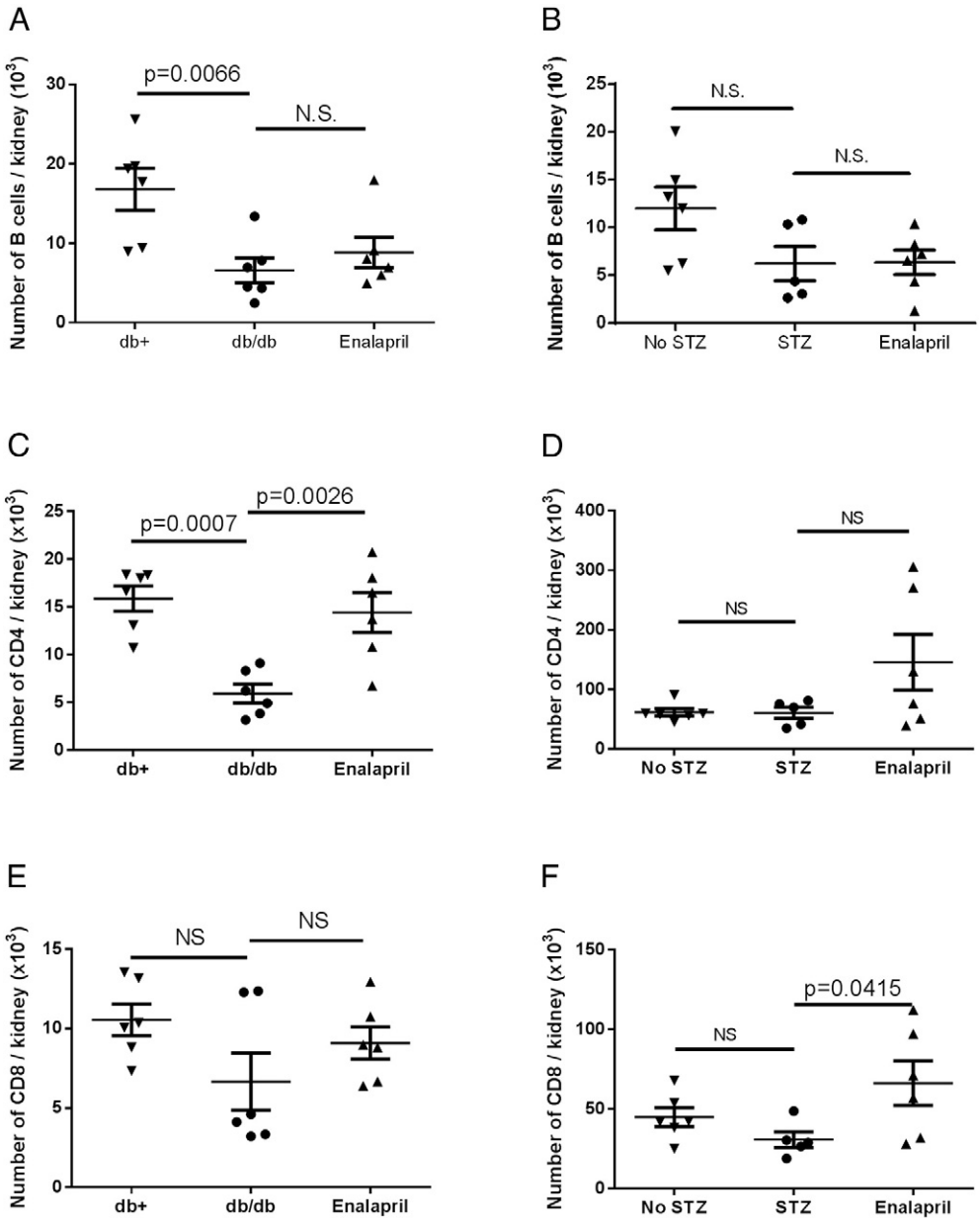

Fig. 7. Total number of B cells (A, B), $CD4^+$ T cells (D, E) and $CD8^+$ T cells (G, H) in db/db (A, C, E) and STZ (B, D, F) kidneys, respectively, with and without treatment with ACE inhibitor and their respective controls (mean ± SEM; n = 6).

lectin, is considered to be a potential central player in the development of DN in man were galectin-3-positive macrophage infiltration in the glomeruli in patients with DN is correlated with progression of the disease [48]. Once there, the macrophage derived galectin-3 is considered to participate strongly in the immunological promotion of renal fibrosis [33]. Pharmaceutical intervention or knock-down of galectin-3 has been shown to render mice less susceptible to STZ induced T1D [48]. However, galectin-3 ablation in a HFD mouse model accelerated adipose tissue and pancreatic islet inflammation probably by favoring development of excessive M1-like macrophages [49]. Furthermore, Pugliese et al. showed that the knock-down of galectin-3 in STZ treated C57Bl/6 mice accelerated diabetes-associated kidney damage rather than providing protection [50]. C57Bl/6 mice are well known to be resistant to diabetic kidney damage, while it is a good strain to induce classical M1 responses [51]. The dose of STZ administrated the frequency of administrations and the length of the model modulates the induction and severity of the disease and could be an explanation for the conflicting results [51]. Still, a consistent feature of galectin-3 is its upregulation in macrophages in chronic inflammation or fibrotic conditions. Galectin-3 is a plieotropic molecule regulating macrophage polarization from M1-like to the more profibrotic M2-like phenotype and strengthening TGFβR signaling by retaining cell surface expression of TGF-β receptors [29,52–55]. In both the db/db and the STZ model, a pronounced expression of TGF-β receptors was noted which might be stabilized due to the upregulated galectin-3 (data not shown). TGF-β production is elevated in peripheral db/db macrophages [22] and herein, a similar increase of TGF-β in kidney derived macrophages in STZ, but not db/db, was noted. Marked increased TGF-β signaling manifested as enhanced pSMAD2/3 has been shown in both db/db and in STZ DN mice models [34,55]. This signaling has been demonstrated to participate to the mesangial cell expansion and the initial fibrotic events occurring in the mice [35]. It is tempting to speculate that the enhanced galectin-3 levels noted together with the presence of TGF-β-TGF-β receptors contribute to the enhanced pSMAD2/3 expression leading to the early fibrotic events.

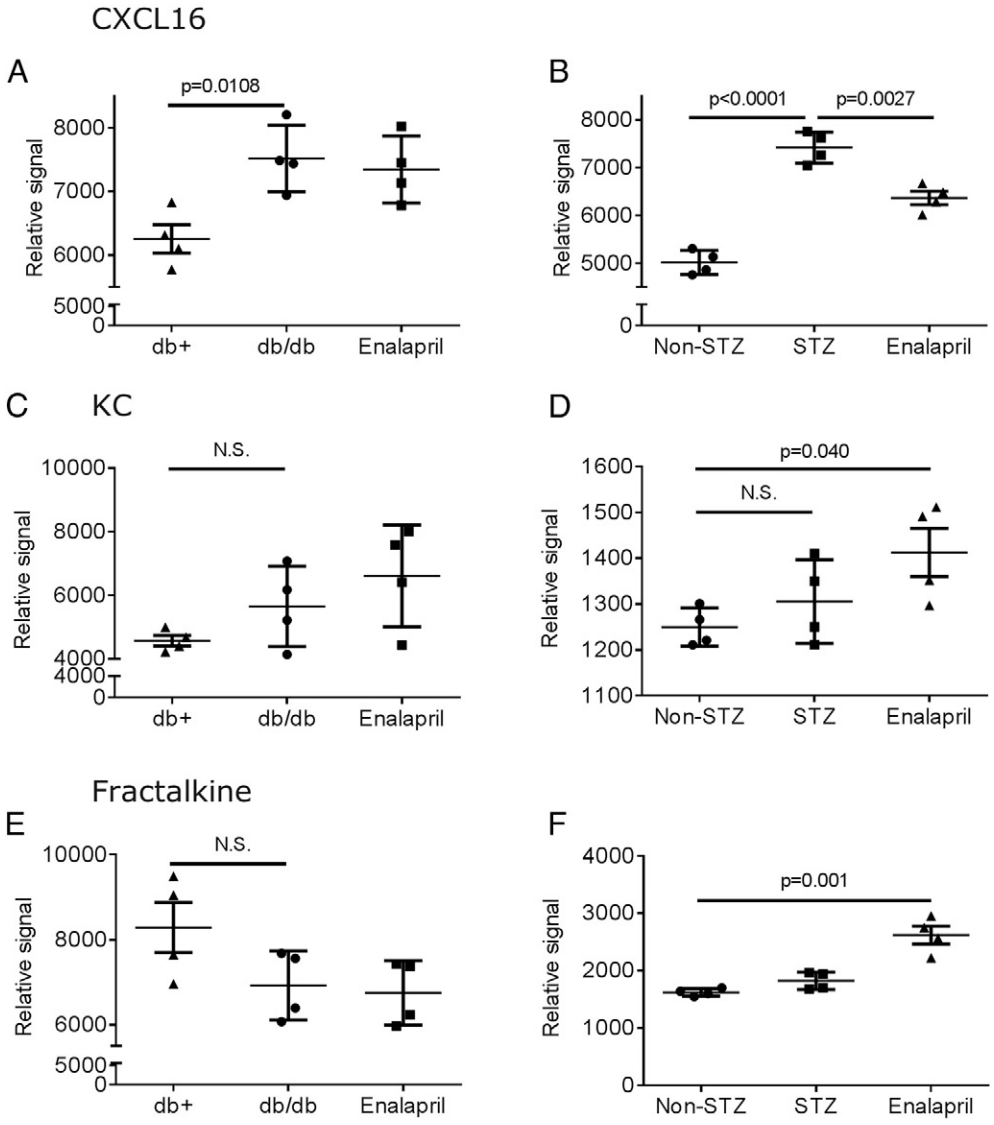

Fig. 8. Expression of cytokines in the diabetic kidney. Relative expression of CXCL16 (A, B), KC (C, D) and fractalkine (E, F) in db/db (A, C, E) and STZ (B, D, F) kidneys, respectively, with or without treatment with ACE inhibitor. Four individual kidneys were evaluated in each condition (mean ± SEM).

Although ACE inhibitors are used as treatment for renal complications, the long term benefit for the patient is only transient and moderate [56]. ACE inhibition is clinically associated with reduction of urinary MCP-1 levels and in pre-clinical models of remnant kidney disease it has been shown to prevent local proliferation of macrophages and myofibroblasts in the kidney [57]. However, Albuquerque et al. showed that enalapril treatment potently increases the number of $CD4^+$ T cells in the spleen together with the increasing production of IL-2 and IL-10 [58]. Our observation that the overall kidney associated leukocyte number, specifically the $CD4^+/CD8^+$ T cells, is increased after enalapril treatment in both db/db and STZ treated mice demonstrates that the angiotensin II blockade overall seems to favor proliferation or attraction of lymphocytes specifically in advanced disease. Furthermore, enalapril treatment modulated the kidney macrophage polarization towards a M1-like phenotype associated with enhanced CD11c, unchanged CD206 and TGF-β expression and reduced galectin-3 expression. This multi-facilitated picture of macrophage polarization stresses the complexity of macrophage plasticity and polarization particularly under pathological conditions *in vivo* [59]. Interestingly, IL-4 and IL-13 stimulation in human macrophages, which promotes M2 polarization, increases ACE expression while LPS and IFN-γ stimulation, which promotes M1 polarization reduce ACE expression in macrophages [60]. Further, Ito et al. elegantly demonstrated that these Th2-associated cytokines (IL-4, IL-13 and PDE2) not only promote the development of M2-macrophages, but also were essential for the fibrotic responses in UUO kidney models in mice [61]. Hence ACE inhibition *in vivo* may counteract production of Th2/M2 associated cytokines thereby promoting a sustained M1 macrophage population. Since enalapril treatment promotes a growing T cell population with a shift in the cytokine signature towards IL-10, with reduced capacity to produce IL-4 and IL-13, it appears like a shift towards a phenotype resembling a T regulatory cell population [62]. This induction of regulatory T cells by ACE inhibitors has previously been shown in autoimmune diseases like multiple sclerosis [62]. Current work in our laboratory is addressing whether enalapril treatment indeed causes a similar development in the diabetic kidney. However, the clinical efficacy of ACE inhibition is moderate [3], similar to the pre-clinical transient beneficial effect observed in this study. This limited effect might be due to the promotion of long-lived M1-like macrophages in diabetic kidney which could contribute to the disease progression despite the polarization of T cells towards a regulatory phenotype.

Taken together, pre-clinical DN is associated with a reduction of immune cells, particularly B and T cells, together with a polarization of the macrophages towards a M2-like phenotype associated with a

significant induction of galectin-3 expression. Enalapril treatment shows a transient efficacy by reducing the albuminuria together with an expansion of the T cells, and by re-polarizing the macrophages towards a M1-like state without modulating the TGF-β expression. This modulation of the local immunity might provide a mechanistical explanation as to why ACE inhibition provides moderate efficacy in man. In conclusion, the data presented provides a first insight to which inflammatory signatures that still remains after the first-line therapy of DN and hence proposes ideas for development of novel therapeutic approaches in the disease.

Authorship

H.C. researched the data, contributed to the discussion and wrote the manuscript. L.N.F. researched the data, contributed to the discussion and wrote the manuscript. M.H.P. researched the data, contributed to the discussion. A.R. conceived the study, researched the data, contributed to the discussion and wrote the manuscript. A.R. is the guarantor of this work and, as such, had full access to all of the data in the study and takes the responsibility for the integrity of the data and the accuracy of the data analysis.

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